ELSEVIER

Biochimica et Biophysica Acta, 1365 (1998) 185–192

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Proton translocation by the respiratory haem–copper oxidases[1]

Mårten Wikström

Helsinki Bioenergetics Group, Department of Medical Chemistry, Institute of Biomedical Sciences and Biocentrum Helsinki, P.O. Box 8, FI-00014 University of Helsinki, Helsinki, Finland

Received 18 February 1998; received in revised form 26 February 1998; accepted 26 February 1998

Abstract

Cytochrome *c* oxidase is a molecular machine that transduces respiratory energy into an electrochemical proton gradient across the mitochondrial or bacterial membrane. Reduction of O_2 to water takes place at a binuclear haem–copper site, to which four electrons are transferred from cytochrome *c* on the outer side of the membrane, and four protons from the inside. An additional and equal contribution to $\Delta\mu_{H^+}$ generation comes from so-called proton pumping, where four H^+ ions are translocated across the membrane per O_2 reduced. Some recent progress in research on the mechanism of energy transduction by this class of enzymes is reviewed. © 1998 Elsevier Science B.V.

Keywords: Cytochrome *c* oxidase; Proton translocation; $\Delta\mu_{H^+}$

1. Introduction

The atomic structures of two cytochrome *c* oxidases have recently been solved [1–4], which has brought the field forward considerably. Although these structures do not yield conclusive functional information, which must come from dynamics, they are of key importance in paving the way for plausible mechanisms as well as for minimizing speculation. Bringing the structural and dynamic experimental approaches together is a major task of today's research in this field.

2. Location of redox centres and generation of $\Delta\mu_{H^+}$

There are four redox-active centres in the enzyme. Cu_A in subunit II functions as a one-electron donor/acceptor and is the entry port of electrons deriving from cytochrome *c* [5]. Three further redox-active metals all reside in subunit I (Fig. 1). Haem *a* accepts electrons from Cu_A and delivers them to the oxygen reduction site. The latter is composed of haem a_3 and a copper ion (Cu_B), which are in close proximity, the so-called binuclear site.

The Cu_A centre lies on the outside of the membrane dielectric [6], which is consistent with the crystal structures. Haem *a* and the binuclear site metals lie ca. one-third of the way into the membrane domain, and at an approximately equal depth (Fig. 1). O_2 diffuses to the binuclear site, possibly through a specific diffusion path in the protein from the centre

[1]If not stated otherwise, all residue numbering in this paper refers to subunit I of cytochrome *c* oxidase from bovine heart mitochondria.

0005-2728/98/$19.00
PII: S0005-2728(98)00060-7

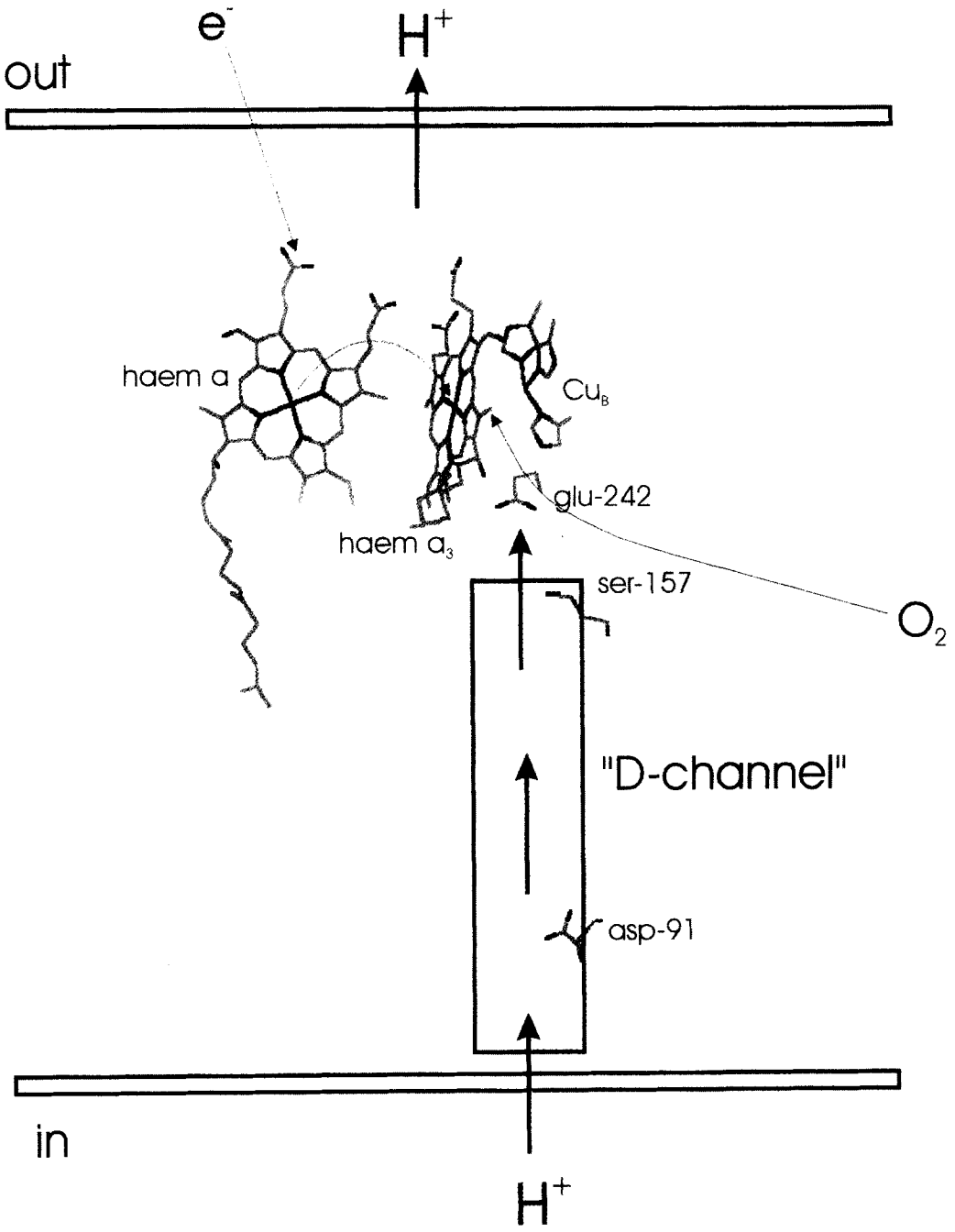

Fig. 1. Scheme of some key structures in subunit I of cytochrome *c* oxidase. *Out* and *in* define the aqueous phases on each side of the membrane into which the enzyme is inserted. Electron transfer is indicated from Cu_A on the outside (not shown) to haem *a*, and further to haem a_3 in the binuclear haem a_3–Cu_B site. O_2 diffusion into this site is shown from the middle of the membrane. The D-channel is a proton-transferring structure that connects the aqueous *in* phase to the invariant Glu-242 (see text). Amino acid numbering is based on cytochrome *c* oxidase from bovine heart mitochondria.

of the lipid membrane [7]. In the overall reaction, which may be written as

$$4e^-_{out} + 8H^+_{in} + O_2 \rightarrow 2H_2O + 4H^+_{out} \quad (1)$$

all protons are taken up from the input side of the membrane, and four of them are consumed in the formation of water. The vectorial arrangement of electron uptake from the outside and proton uptake from the inside in the oxygen reduction chemistry generates $\Delta\mu_{H^+}$. This partial function is conceptually analogous to the arrangement of electron and proton transfer reactions in bacterial and plant photosynthesis, all examples of Peter Mitchell's notion of 'vectorial metabolism' [8]. However, four more protons are translocated across the membrane per O_2 reduced. This so-called proton pumping function [9] doubles the overall conservation of energy by the enzyme, and represents quite a different type of $\Delta\mu_{H^+}$ generation conceptually. Despite this, proton uptake for consumption and for translocation may be tightly intertwined both mechanistically and thermodynamically in the haem–copper oxidases [10–12]. Understanding the mechanism of proton translocation in respiration and for bacteriorhodopsin [13] is presently one of the key problems in bioenergetics.

3. The D-channel

Two proton-conducting pathways (D- and K-channels) have been proposed in subunit I of the haem–copper oxidases, each named after a key residue in the respective structure (Asp-91 and Lys-319). A third pathway between helices IX and XII of subunit I was proposed on the basis of the crystal structure [3], but has not received functional support [14]. A proton entry domain of the D-channel (Fig. 2) was first identified by site-directed mutagenesis experiments: Non-conservative mutations of Asp-91[1], Asn-80 and Asn-98 yielded decoupling of proton translocation from electron transfer in the bo_3-type quinol oxidase of *Escherichia coli* cells [15,16]. The role of Asp-91 in proton translocation was subsequently confirmed in reconstituted aa_3- and bo_3-type enzymes from *Rhodobacter sphaeroides* [17] and *E. coli* [18], respectively. The carboxylic group of Asp-91 need not be located in this precise position, but can be moved to two other unique positions in the domain (replacing Pro-95 and Asn-98) without loss of proton-translocating efficiency [16]. This finding is consistent with the crystal structure [3], which shows proximity of Asp-91, Pro-95 and Asn-98 (Fig. 2), and suggests that a key property of the channel entry may be to provide attraction for protons. Interestingly, this domain is near the interface between subunits I and III. The crystal structures identified a likely proton transfer path from this domain towards the middle of the membrane, lined by mostly polar residues [1,3,4], i.e. the D-channel (Fig. 1). This path also contains several bound water molecules, identified both from calculations ([19,20]; Fig. 2) and from the crystal structures, and ends near Ser-157, in a cavity near the middle of the membrane (Figs. 1 and 2). Proton transfer may therefore be mediated by water molecules and modulated by the amino acid side chains, as described in simulations of proton transfer in the gramicidin channel [21]. Mutation of Ser-157 (Ser-201 in aa_3 of *R. sphaeroides* and Thr-201 in cytochrome bo_3) to a small residue does not affect proton translocation efficiency [19,22], presumably because the water molecules in its vicinity can easily rearrange. However, mutation to Phe decouples proton-pumping [19], providing functional support for the notion that the D-channel is used in proton translocation in its entirety.

We have preferred to restrict the D-channel nomenclature to the structure from the inside of the membrane to the Ser-157 domain (Fig. 1), while others include Glu-242 as part of it. Our preference is based on the crystal structures and the special role of Glu-242 (see below). The X-ray structures do not identify any protonic connectivity onwards from Ser-157, although Iwata et al. [1] suggested that bound waters might connect it further to the invariant Glu-242. In contrast, Tsukihara et al. [3] proposed further connectivity towards the OUT domain by a channel between helices III and IV, with no connection to Glu-242. This channel is not well conserved, however.

On the other hand, calculations predict an array of ca. three water molecules connecting the D-channel to the Glu-242 side chain [19,20]. Mutation of Glu-242 to Cys, but not to Asp, in the reconstituted bo_3 enzyme, decouples proton translocation [18], which provides strong functional evidence for the notion that the D-channel indeed connects to Glu-242. Other

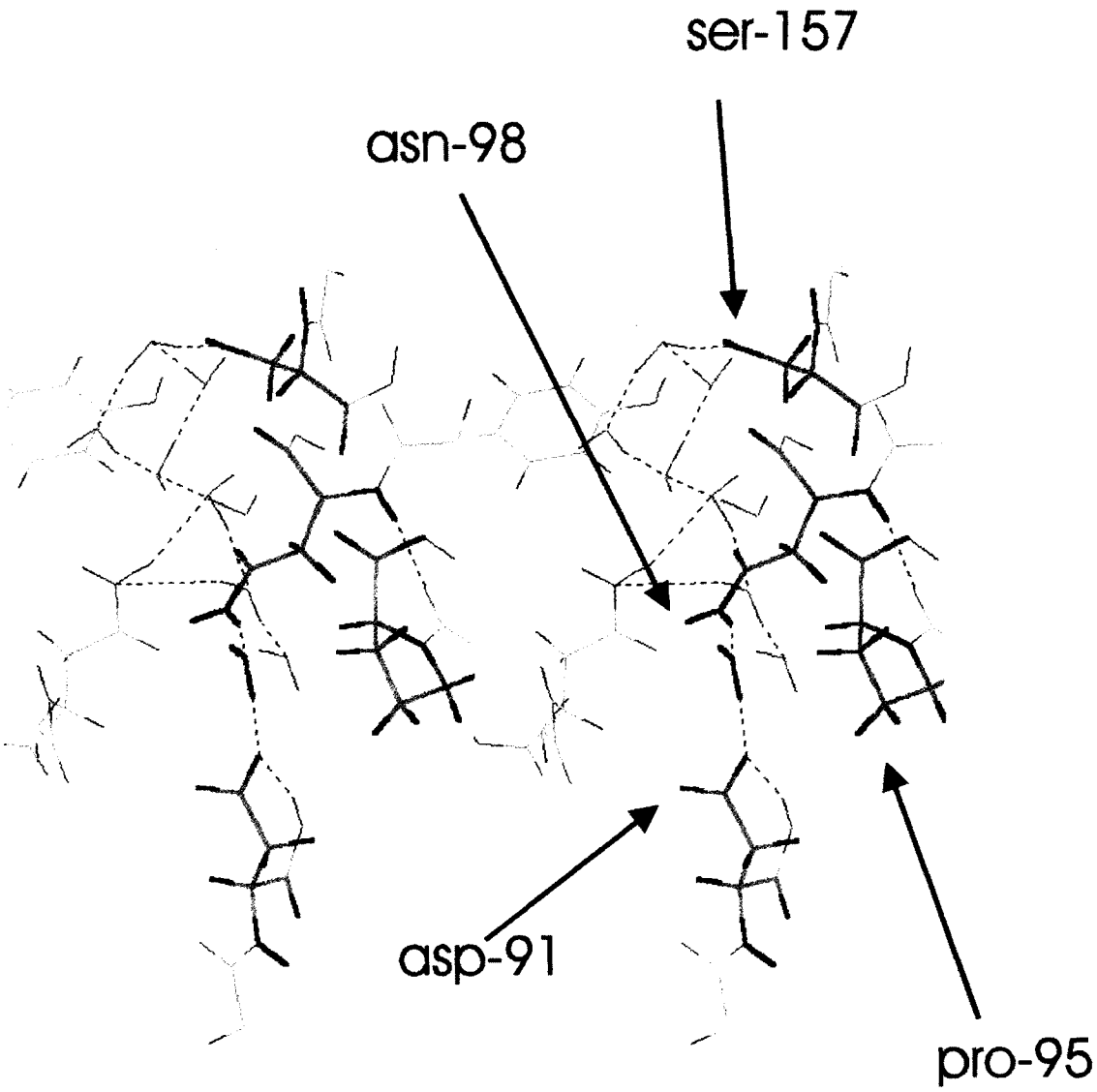

Fig. 2. Entrance of D-channel. Stereo view of some key residues near the entrance, and of Ser-157 at the exit (see Fig. 1). Predicted water molecules in the channel are also shown [19]. Side chains marked with arrows, and a water molecule between Asp-91 and Asn-98, are high-lighted. The side chains of Asn-80 (near Asn-98) and Tyr-19 are also shown.

mutations of Glu-242 have been shown to block the oxygen reduction chemistry as well as proton uptake by the isolated enzyme [23,24].

4. The role of Glu-242 — a molecular ratchet?

Both experimental and theoretical considerations suggest that the glutamic acid side chain may have to undergo conformational isomerisations between the crystallographic IN position and an OUT position in order to shuttle protons further towards the outside of the membrane [19,20]. Three residues that might accept protons from Glu-242 in an OUT conformation have been proposed thus far, viz. the εN of the Cu_B ligand His-291 [19,25,26], a propionate of haem a_3 [20] and oxygen bound to the binuclear site ([25,26]; see below). However, the crystal structures have not to date revealed further connectivity beyond an OUT conformation of the Glu-242 side chain, which does not as such reach any of the proposed acceptor moieties. Calculations ([19,20]) suggest that

this connectivity may be provided by bound water molecules in this region. In addition, Puustinen et al. [27] showed by FTIR spectroscopy that there may be a polarisable connectivity between the protonated and strongly hydrogen-bonded side chain of Glu-242 and a histidine ligand of Cu_B in the reduced bo_3 enzyme. This is consistent with connectivity between Glu-242 and Nε of the Cu_B ligand His-291 via water molecules that were independently predicted to span between these moieties [19]. With small rearrangements, the same water molecules could also connect the OUT position of the Glu-242 side chain to the Δ-propionate of haem a_3 (see also [20]), which Iwata et al. [1] originally considered to be involved in proton translocation, and for which we have suggested a key role in the histidine cycle model as a possible interaction partner with a moving histidine ligand of Cu [25].

Engineering a lysine or an arginine into the vicinity of Glu-242 leads to loss of proton translocation [19]. However, this does not occur in the Glu-242–Asp mutant (S. Riistama, unpublished), which, as such, exhibits normal proton translocation efficiency and only slightly reduced turnover [18]. Thus, a positively charged side chain in this domain is not particularly destructive per se, unless it can interact directly with the carboxyl group. This occurs with Glu-242 but not with the shorter side chain of Asp-242, which is consistent with the idea that the carboxylic side chain may have to move during proton translocation. Pomès et al. (this volume) recently performed molecular modelling of the protonated and unprotonated side chain of Glu-242. These calculations reveal that rotational isomerisation between IN (crystal structure) and putative OUT configurations is both thermodynamically and kinetically feasible. Moreover, the carboxylate anion seems to be much less stable in the OUT than in the IN state, while the stability of the acid form is similar in these states. This may be ideal for a proton-transferring site that functions as a molecular ratchet, preventing thermodynamically favourable back-leakage of protons kinetically.

Conformational isomerisation of the Glu-242 side chain between IN and OUT positions is of great interest with regard to the proton pump mechanism. This is precisely the kind of property expected from a key acid/base element of such a mechanism [28,29]. Further work is required to assess if the redox and ligand state of the binuclear site influences the shuttling of Glu-242. If significant, then another key property of the 'pump element' would be satisfied, viz. that of linkage to the driving chemistry [28,29]. Two facts are presently difficult to reconcile with such a function of Glu-242, however. One is the proton translocation stoichiometry of 2 H^+/e^- for the P→F and F→O steps, respectively ([30]; Fig. 3); the other is the finding that the D-channel (plus Glu-242) delivers both pumped and consumed protons.

5. Critical assessment of the function of the D- and K-channels

Fig. 3 shows a scheme of the catalytic cycle defining the two phases of the reaction that might utilise the two proton channels, the roles of which are extensively reviewed in this issue (e.g. Karpefors et al.). It is less frequently pointed out, however, that functional evidence for the 'K-channel' as a proton-transferring pathway is indirect. Mutation of the key residue K-319 blocks reduction of haem a_3 ([23]; step E→R, Fig. 3), and impairs proton transfer-coupled electron transfer backwards from haem a_3 to a [31], but reduction of Cu (step O→E) may still be fast [32]. This is not entirely consistent with the notion that both protons taken up on enzyme reduction are transferred through the K-channel. Jünemann et al. [32] suggest that the K-channel may instead function as a 'dielectric well', and Rottenberg [33] has proposed that it transfers OH anions from the binuclear site. There is no evidence for its participation in proton pumping. On the contrary, in the Lys-319–Met mutant, the electron transfer kinetics and proton uptake in the oxidative half of the catalytic cycle are unaffected [31,34], although later phases of proton uptake may be somewhat decelerated (see Karpefors et al., this volume). Also, the 'peroxidase cycle' (see Fig. 3) is associated with proton-pumping in this mutant [35,36].

Apart from conducting the pumped protons, the D-channel almost certainly conducts those protons that are consumed in the O_2 reduction chemistry, whose net uptake takes place after O_2 binding in the catalytic cycle ([23,24,34,35,37,38]; Fig. 3). Therefore, there must also be protonic connectivity beyond

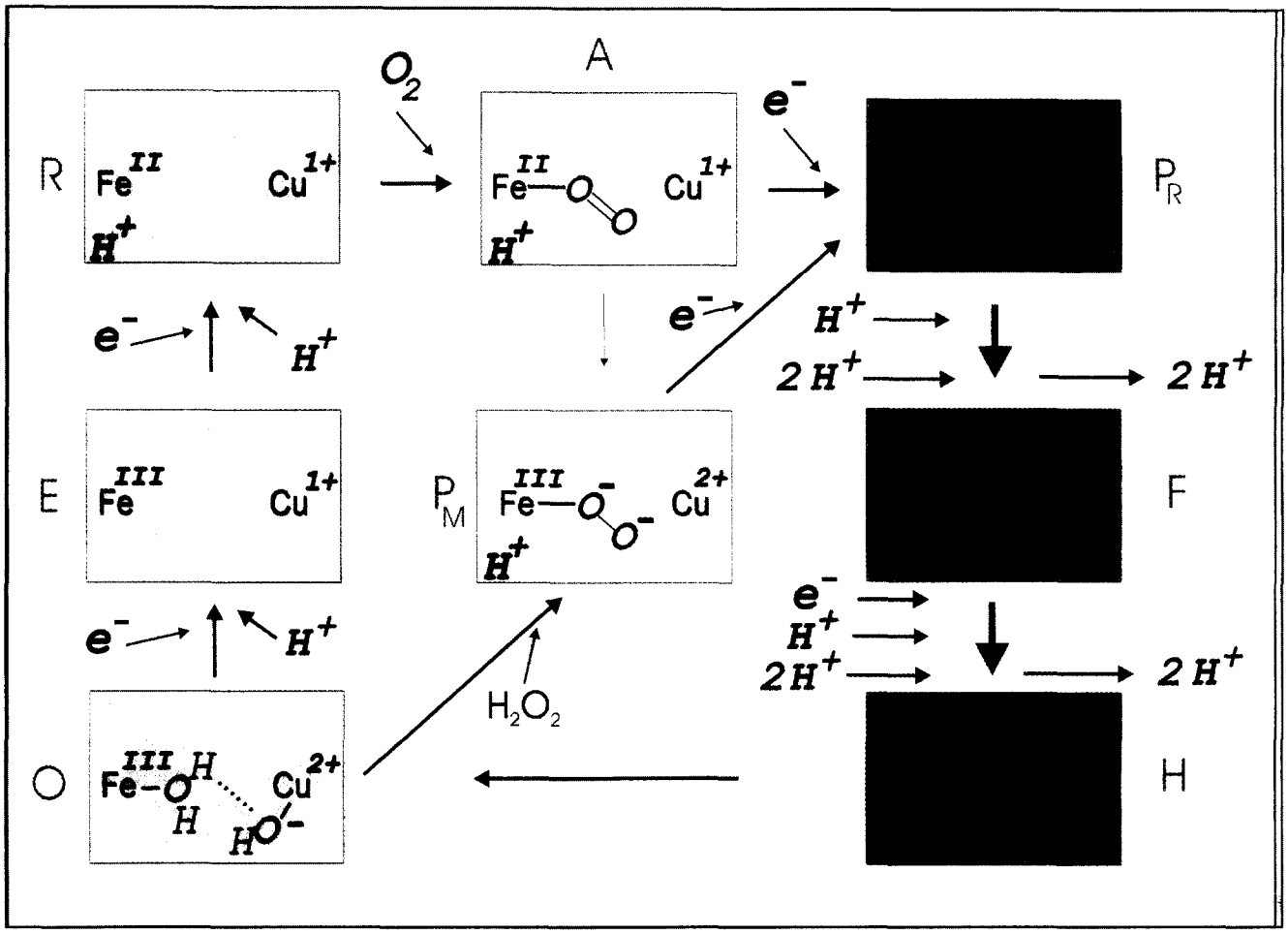

Fig. 3. Model of the catalytic cycle. The main cycle is clockwise from state O through states E, R, A, P_R, F and H. O→R denotes reduction of the binuclear site, accompanied by proton uptake from the inside of the membrane. After binding of O_2 (state A), the site can quickly receive an electron from haem *a* (ca. 30×10^3 s^{-1}) to form the P_R state. State A decays more slowly to the P_M state (ca. 5×10^3 s^{-1}) if no electron is available in haem *a*. The sequence $P_R(P_M)\rightarrow F\rightarrow H\rightarrow O$ represents the 'power stroke', which is coupled to the major energy-dependent steps of proton translocation [30]. Key intermediates in this process are coloured red, as is the associated proton uptake from the inside (left) and release to the outside (right) of the dielectric barrier. The 'peroxidase cycle' is initiated by state O, which reacts with added H_2O_2, by which P_M is formed (see text). The structures of Fe_{a3} and Cu_B are presently under debate, especially for states P_M and P_R, which are identical by optical spectroscopy, but very different from state F.

the Glu-242 to the binuclear site, presumably via the bound water molecules (cf. above).

However, we must still remain cautious about the precise role of the two proton channels. Consider for example the following scenario: The fate of the proton taken up in the E→R step of the cycle (presumably via the K-channel) is not known (Fig. 3). It may not necessarily be consumed in O_2 reduction to water, but could subsequently be translocated across the membrane in the steps P→O. This may not be revealed in K-channel mutants; neither in flow/flash experiments starting from the pre-reduced (and pre-protonated) enzyme, nor from experiments studying the 'peroxidase cycle', where this proton could be taken up from the added H_2O_2, thus by-passing the channel. It should be emphasised that such a scenario is also consistent with the finding that the O_2 reduction phase of the catalytic cycle (P→O; Fig. 3) is linked to proton translocation [30]. This work showed that the O_2 reduction phase (P→O) must be associated with proton translocation on thermodynamic grounds. However, since we do not

know the fate of the protons taken up in the O→R step, one or even both of them could still end up on the other side of the membrane in subsequent steps.

6. Implications for the pump mechanism

In my view, it is very important mechanistically that the D-channel (and Glu-242) can be shown to transfer *both pumped and consumed protons*. This not only implies that the strongly exergonic uptake of protons to be consumed in the O_2 reduction chemistry can be utilised directly to drive the proton pump [10–12,25,26], but also points at the necessity of a key control or gating system, which passes these protons either for pumping or for consumption [25,26]. A simple way to achieve this is that, in its INPUT position, the pump element itself blocks proton access into the binuclear site. This could assure primary protonation of this element, after which, its switch to an OUTPUT position would open proton access to the binuclear site. We have proposed that the Cu_B ligand His-291 might have such a function [25,26].

The question has often been asked about how it is possible that the turnover of the bo_3 quinol oxidase enzyme from *E. coli* is inhibited only by some 50% in D-channel mutants, while proton-pumping appears to be entirely abolished in multi-turnover experiments [15,16,18]. In contrast, turnover of corresponding mutant enzymes from *R. sphaeroides* [17] and *P. denitrificans* [14] are >90% inhibited. One must recall, first, that the reactions comprising the P→O steps are much faster than those comprising O→R (Fig. 3). Thus, partial inhibition of the former need not block turnover to any great extent. Moreover, proton translocation can be partially recovered in the Asp-91 mutant of bo_3 at very low pH of the IN phase (M. Verkhovskaya, unpublished data). It follows that kinetic delay of proton transfer through the D-channel impairs the efficiency of the proton pump. We believe that this may be due to a kinetic failure of the proton gating system, discussed above, which normally distributes D-channel protons both for pumping and for consumption.

Fetter et al. [17,39] found 'reversed respiratory control' in a D-91 mutant of the *R. sphaeroides* aa_3 enzyme. This was interpreted as $\Delta\psi$-driven proton uptake from the outside aqueous phase to the binuclear site via the output path of the pump. However, there is at least one alternative interpretation, which does not involve the pump's exit path. An input conformation of the pump may still be protonated from the D-channel, even though kinetically impaired by the mutation, but since protonation of the binuclear site is very slow, the pump cannot deliver the proton(s) into the exit path. Instead, a leak path becomes favoured from the protonated pump element to the binuclear site, which may well be accelerated two-fold by $\Delta\psi$ causing 'reversed respiratory control'. In this scenario, the small effect of the D-91 mutation on the turnover of the bo_3 enzyme, relative to aa_3, can be explained simply by a larger a priori rate constant of this leak path in bo_3, which is consistent with the unique tendency of this enzyme to become decoupled at high pH [40].

Note that both alternative explanations of 'reversed respiratory control' imply that the pump element is close to the binuclear site. Therefore, the mechanism of coupling O_2 reduction to proton translocation is likely to be a relatively direct one, involving the cofactors and/or ligands of the binuclear site, or at least residues in their vicinity.

Acknowledgements

This work was supported by grants from the University of Helsinki, the Academy of Finland, Biocentrum Helsinki and the Sigrid Jusèlius Foundation. I would like to thank the members of my research group for their excellent experimental work and for discussions.

References

[1] S. Iwata, C. Ostermeier, B. Ludwig, H. Michel, Nature 376 (1995) 660–669.
[2] T. Tsukihara, H. Aoyama, E. Yamashita et al., Science 269 (1995) 1017–1188.
[3] T. Tsukihara, H. Aoyama, E. Yamashita et al., Science 272 (1996) 1136–1144.
[4] C. Ostermeier, A. Harrenga, U. Ermler et al., Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 10547–10553.
[5] B.C. Hill, J. Biol. Chem. 266 (1991) 2219–2226.

[6] M. Erecinska, B. Chance, D.F. Wilson, FEBS Lett. 16 (1971) 284–286.
[7] S. Riistama, A. Puustinen, A. García-Horsman et al., Biochim. Biophys. Acta 1275 (1996) 1–4.
[8] P. Mitchell, in: G. Semenza (Ed.), Of Oxygen, Fuels, and Living Matter, Part 1, Wiley, Chicester, New York, 1981, pp. 1–56.
[9] M. Wikström, Nature 266 (1977) 271–273.
[10] M. Wikström, A. Bogachev, M. Finel et al., Biochim. Biophys. Acta 1187 (1994) 106–111.
[11] J.E. Morgan, M.I. Verkhovsky, M. Wikström, J. Bioenerg. Biomembr. 26 (1994) 599–608.
[12] P.R. Rich, Aust. J. Plant Physiol. 22 (1995) 479–486.
[13] U. Haupts, J. Tittor, E. Bamberg et al., Biochemistry 36 (1997) 2–7.
[14] U. Pfitzner, A. Odenwald, T. Ostermann et al., J. Bioenerg. Biomembr. 30 (1998) 89–97.
[15] J.W. Thomas, A. Puustinen, J.O. Alben et al., Biochemistry 32 (1993) 10923–10928.
[16] J.A. García-Horsman, A. Puustinen, R.B. Gennis et al., Biochemistry 34 (1995) 4428–4433.
[17] J.R. Fetter, J. Qian, J. Shapleigh et al., Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 1604–1608.
[18] M.L. Verkhovskaya, A. García-Horsman, A. Puustinen et al., Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 10128–10131.
[19] S. Riistama, G. Hummer, A. Puustinen et al., FEBS Lett. 414 (1997) 275–280.
[20] I. Hofacker, K. Schulten, Proteins Struct. Funct. Genet. 30(30) (1998) 100–107.
[21] R. Pomès, B. Roux, Biophys. J. 71 (1996) 19–39.
[22] D.M. Mitchell, J.R. Fetter, D.A. Mills et al., Biochemistry 35 (1996) 13089–13093.
[23] M. Svensson-Ek, J.W. Thomas, R.B. Gennis et al., Biochemistry 35 (1996) 13673–13680.
[24] P. Ädelroth, M. Svensson-Ek, D.M. Mitchell et al., Biochemistry 36 (1997) 138–13829.
[25] M. Wikström, J.E. Morgan, G. Hummer, et al., in: S. Papa, F. Guerrieri, J.M. Tager (Eds.), Frontiers of Cellular Bioenergetics, Plenum Press, New York, in press.
[26] M. Wikström, J.E. Morgan, M.I. Verkhovsky, J. Bioenerg. Biomembr., in press.
[27] A. Puustinen, J.A. Bailey, R.B. Dyer et al., Biochemistry 36 (1997) 13195–13200.
[28] M. Wikström, K. Krab, Biochim. Biophys. Acta 549 (1979) 177–222.
[29] M. Wikström, K. Krab, M. Saraste, Cytochrome Oxidase-A Synthesis, Academic Press, New York and London, 1981, pp. 144–145.
[30] M. Wikström, Nature 338 (1989) 776–778.
[31] P. Brzezinski, P. Ädelroth, J. Bioenerg. Biomembr. 30 (1998) 99–107.
[32] S. Jünemann, B. Meunier, R.B. Gennis et al., Biochemistry 36 (1997) 14456–14464.
[33] H. Rottenberg, Biochim. Biophys. Acta, in press.
[34] M. Svensson, S. Hallén, J.W. Thomas et al., Biochemistry 34 (1995) 5252–5258.
[35] A.A. Konstantinov, S. Siletsky, D. Mitchell et al., Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 9085–9090.
[36] T.V. Vygodina, N. Capitanio, S. Papa et al., FEBS Lett. 412 (1997) 405–409.
[37] M. Oliveberg, S. Hallén, T. Nilsson, Biochemistry 30 (1991) 436–440.
[38] R. Mitchell, P.R. Rich, Biochim. Biophys. Acta 1186 (1994) 19–26.
[39] J.R. Fetter, M. Sharpe, J. Qian et al., FEBS Lett. 393 (1996) 155–160.
[40] M. Verkhovskaya, M. Verkhovsky, M. Wikström, J. Biol. Chem. 267 (1992) 14559–14562.